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TECHNICAL MANUSCRIPT 252

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SERUM TITRATION OF HUMAN AND ANIMAL COCCIDIOIDOMYCOSIS BY AGAR-GEL PRECIPITIN INHIBITION

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NOVEMBER 1965

UNITED STATES ARMY
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SERUM TITRATION OF HUMAN AND ANIMAL COCCIDICIDOMYCOSIS BY AGAR-GEL PRECIPITIN INHIBITION

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Medical Investigation Division
DIRECTORATE OF MEDICAL RESEARCH

Project 1C622401A072

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In conducting the research reported here, the investigators adhered to "Principles of Laboratory Animal Care" as established by the National Society for Medical Research.

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ABSTRACT

An application of the agar-gel precipitin-inhibition technique of Ray and Kadull detects Coccidioides immitis antibodies in sera from human clinical cases and experimental animals infected with this organism.

The advocated modification of that agar-gel procedure can detect this antibody more consistently and reliably than the complement-fixation and double immunodiffusion tests.

I. INTRODUCTION

The complement-fixation (CF) test is the standard serological test for coccidioidomycosis. However, it is subject to variable results, as shown by Huppert and Bailey[1] and Converse et al.[2] who respectively found nonspecific low-level CF titers in sera from humans and from monkeys.

Recently an immunodiffusion (ID) test was reported and recommended by Huppert and Bailey[1] as a screening test for this disease. This test was easily adapted to laboratory routine and was a more specific indicator of coccidioidomycotic infections than the CF test. The ID test, however, revealed only the presence of positive or negative sera; no titration or definite titers were assigned the sera because of multiple line formation.

The present investigation was undertaken to ascertain whether the agar-gel precipitin-inhibition (AGPI) test could determine antibody titers that would compare with those measured by the CF test in known cases of human and experimental animal coccidioidomycosis. If correlative, this procedure would provide a definite titration of the positive coccidioidomycotic serum, and it would eliminate the additional components used in the CF test method such as complement or an indicator system (sensitized sheep red blood cells). These latter components, as well as the test procedures, varied greatly in a previously reported coccidioidomycotic CF test inter-laboratory evaluation by Smith et al.[3]

In addition, the AGPI test was compared with a modified ID agar-gel procedure. The ID test of Huppert and Bailey[1] was modified in this investigation so that similar conditions of diffusion of the Coccidioides antigen and antibody would prevail in similarly prepared agar-gel plates.

II. MATERIALS AND METHODS

A. MONKEY COCCIDIOIDAL ANTISERUM

Sera from monkeys aerogenically exposed to Coccidioides immitis, Cash strain, and having a CF titer of 1:512, were used in the test procedure.

B. COCCIDIOIDIN ANTIGEN PREPARATION

The coccidioidin antigen used for the AGPI test was kindly prepared with C. immitis, Cash strain, and supplied by Mr. Gordon Taylor.[4]

C. AGAR-GEL MEDIUM AND PLATES

The test medium and plates were prepared as described by Ray and Kadull.[5]

D. AGAR-DIFFUSION READING LAMP

The reading lamp was the same as that used by Ray and Kadull.[5]

E. BLOCK TITRATION OF THE ANTIGEN-ANTIBODY SYSTEM

Five serial twofold dilutions of the positive serum and of the antigen were prepared separately in physiological saline beginning with 0.5 ml of the antigen and 0.2 ml of the antiserum. To each dilution an equivalent volume of physiological saline was added to give final dilutions ranging from 1:2 to 1:32. The center row of reservoirs (approximately 0.025 ml volume per reservoir) was filled with one of the prepared antiserum dilutions, one plate being used for each dilution. Plates were incubated at room temperature, 23 to 27 C, for 1 hour. Following incubation, each of the two outer rows of wells (approximately 0.07 ml volume per well) was filled sequentially and in duplicate with the coccidioidin antigen dilutions.

Plates were observed with the aid of the previously described visual apparatus after remaining at room temperature for 24 hours; the final reading occurred at 48 hours.

The antigen-antibody end point was determined as that combination of the highest dilution of antigen and antibody that produced a visible line of precipitate in 48 hours. This initial titration in the agar-gel plates was essential to the establishment of maximum sensitivity and consistent reproducibility in subsequent tests with unknown sera. The end point reading was arbitrarily assumed to represent a minimum reacting dilution (MRD_a) of coccidioidin antigen, and a minimum reacting dilution (MRD_s) of antibody to coccidioidomycosis (Table 1). The MRD_a of antigen was established as a 1:128 dilution and the MRD_s of the antibody as a 1:16 dilution.

F. SERUM TITRATIONS

The inhibition, or indirect, method was used in titrating unknown sera. Serial twofold dilutions of 0.2 ml of unknown serum were made in physiological saline. To each dilution, 0.2 ml of the previously block-titered antigen (1:128) was added; the final mixture thus contained 1/2 MRD_a plus unknown serum dilutions ranging from 1:2 to 1:32. The mixtures were handshaken for 10 seconds and incubated in a 37 C water bath for 30 minutes to permit antigen-antibody binding to proceed to completion.

Each of the wells in the center row of agar diffusion plates was filled with a 1:16 dilution (MRD_s) of monkey antiserum and the plates were incubated at room temperature, 23 to 27 C, for 1 hour. Conclusions of incubation periods for the plates and for the antigen-antibody binding mixture were timed to coincide and permit immediate completion of the final step.

TABLE 1. COCCIDIOIDAL ANTIGEN-ANTIBODY AGAR-GEL BLOCK TITRATION

Monkey Serum Dilution, 1:512 CF titer	Coccidioidin Dilution					
	1:8	1:16	1:32	1:64	1:128	1:256
1:2	+	+	+	+	±	-
1:4	+	+	+	+	±	-
1:8	+	+	+	+	+	-
1:16	+	+	+	+	+	-
1:32	±	±	-	-	-	-

Outer rows of wells were filled sequentially and in duplicate with the incubated antigen-serum dilution mixtures. Thus, each well in one outer row contained the same mixture as the corresponding well in the opposite outer row. The end point was determined after plates remained at room temperature for 48 hours. A preliminary 24-hour reading was generally made. The end point, or titer, of an unknown positive serum was the dilution that completely inhibited the formation of a visible line of precipitate with the block-titrated MRD_s and MRD_s in the agar-gel plates (Fig. 1).

Controls subjected to the same test procedure consisted of dilutions of the test antigen in saline and combinations of the predetermined MRD_s of antigen with negative and positive coccidioidomycotic sera.

Figure 1. Serum Titrations by the AGPI Technique.
(Top) Negative serum: Note undulating lines of visible precipitate between center and outer rows of wells, indicating absence of inhibition of the coccidioidin MRD_a.

(Bottom) Positive serum: Note absence of visible line of precipitate between the first three wells on the left of center and outer rows, indicating complete binding of coccidioidin MRD_a; titer end point is 1:8 dilution of the serum.

G. IMMUNODIFFUSION TEST FOR COCCIDIOIDOMYCOSIS

The direct immunodiffusion (ID) test was performed by placing concentrated and 1:10 dilutions of coccidioidin antigen in the center wells. After a prior 30-minute incubation period at room temperature, serial twofold dilutions of known CF-positive monkey antiserum were placed in the outer series of wells, sequentially and in duplicate. These plates were allowed to incubate at room temperature for 48 hours, at which time the final titer of the serum was recorded. Initial reactions in high-titered sera were evident as early as 6 hours.

H. COMPLEMENT-FIXATION TEST FOR COCCIDIOIDOMYCOSIS

When the complement-fixation test (CF) was performed on sera specimens, the procedure used was that described by Smith et al.[8] The overnight fixation procedure was employed in these determinations. Because of the high percentage of anticomplement activity, monkey and human sera were either pretreated with complement, as described by Wadsworth[7] or heated for 1 hour at 56 C in an attempt to eliminate this unwanted characteristic.

The coccidioidin CF antigen and positive human CF control serum were obtained from the Diagnostic Reagents Section, Communicable Disease Center, Atlanta, Georgia, and were from Lot 8 of 9-1-61 and Lot 5 of 7-17-61 respectively.

III. RESULTS

A. COMPARISON OF CF, ID, AND AGPI TITERS ON MONKEY SERA

Forty-five monkey sera were serologically titrated in physiological saline and directly diffused in agar-gel plates against concentrated and 1:10 dilutions of coccidioidin antigen. Then AGPI titers were determined on these same sera and these results were compared with the previously determined CF titers. Twenty representative monkey sera results are presented in Table 2 and portray the variation in serological test findings. These results indicate that the AGPI technique is more sensitive and attains higher titers than the ID technique. It is not as sensitive as the CF test, although titers were generally within a one-tube variance.

TABLE 2. COMPARISON OF THE CF, ID, AND AGPI SEROLOGICAL TESTS FOR COCCIDIOIDOMYCOSIS IN 20 MONKEY SERA AFTER RESPIRATORY CHALLENGE WITH VIABLE ARTHROSPORES

Monkey Serum Number	Reciprocal ID Titer		Reciprocal AGPI Titer	Reciprocal CF Titer
	Conc.	1:10		
S-12	-	-	-	5
S-50	-	-	4	5
T-61	-	-	-	-
B-3	-	-	4	20[a]
S-36	-	-	2	10
S-30	2	2	8	80
B-172	2	2	16	80[a]
J1-6	2	2	32	80
B-22	2	2	32	40
B-104	4	4	128	160[a]
S-12	4	4	128	160
A-5	4	4	128	80
T-62	4	8	128	320[a]
R-31	4	8	256	320
S-52	16	16	256	320
L2-9	4	16	512	320
S-40	16	32	512	2560
B1-30	32	32	1024	640
6C-17	32	32	2048	2560
S-6	32	64	4096	5120[a]
Positive Human Serum Control CF titer 1:64 Lot 5 (7-17-61)	4	8	64	80

a. Serum still retained anticomplement activity.

B. COMPARISON OF CF AND AGPI TITERS ON HUMAN SERA

Seventeen human sera from clinically diagnosed cases of coccidioidomycosis were obtained from various laboratories: six from the Walter Reed Army Institute of Research (WRAIR), six from the National Institutes of Health (NIH), and nine from the University of California. All sera were titrated by the CF method at the donor institutions, except that the NIH sera were titrated by the WRAIR personnel. Table 3 shows the results obtained by the AGPI technique on these sera and compares these titers with those obtained by the institutional CF techniques. Generally, the titer variation between these two serological techniques averaged only one tube dilution, and was almost consistently one tube titer less in the AGPI test especially when compared with the WRAIR CF technique.

TABLE 3. COMPARISON OF TITERS BY THE COMPLEMENT-FIXATION AND AGAR-GEL PRECIPITIN-INHIBITION TECHNIQUES ON HUMAN SERA

Source	Serum No.	Source Reciprocal CF Titer	AGPI Reciprocal Titer
University of California	H-9653	512	256
	H-1097	64	64
	H-4262	128	64
	H-4684	16	16
	H-4782	2	8
	H-5387	16	16
	H-5675	128	128
	H-5703	4	16
	H-5765	8	32
National Institutes of Health	B-10468	128	32
	B-4802	128	64
	B-10989	32	16
	B-12838	32	8
	B-13041	32	16
	B-8690	32	8
Walter Reed Army Institute of Research	184	-	-
	315	32	32
	1	32	16
	4	128	32
	5	32	16
	6	-	-

C. COMPARATIVE SEROLOGICAL RESPONSE OF MONKEYS TO CUTANEOUS AND PULMONARY COCCIDIOIDOMYCOSIS

Several groups of monkeys were subcutaneously vaccinated with live Silveira strain and formalin-killed Cash strain C. immitis spores. These monkeys were subsequently challenged by the respiratory route with C. immitis Silveira strain, as previously reported by Converse et al.[8] Serial serum samples were obtained and titered by the CF and AGPI techniques in an effort to determine if the AGPI test would detect an antibody response to coccidioidomycosis earlier and more consistently than the CF test. The results of the experiment are presented in Table 4. This table shows the comparison between the CF and AGPI test results on immunized and nonimmunized monkey sera, before and 15 days after a viable respiratory challenge, and indicates the greater sensitivity of the AGPI method in these titer determinations. The AGPI titers persisted longer than the CF titers in the viable-vaccinated monkeys and mirrored the positive skin test reactions prior to the respiratory challenge. After challenge, these results indicate a general rise in titer by both test methods in the viable-vaccinated group, but only the AGPI technique detected a similar rise in the nonviable-vaccinated group of monkeys.

D. SPECIFICITY OF THE AGPI TEST FOR COCCIDIOIDOMYCOSIS

It was apparent from the above experiments that the AGPI test procedure was serologically sensitive enough to determine antibody titers in monkey and human sera. Since the antigen employed in the AGPI test was derived from the Cash strain of C. immitis, and the experiments contained antisera from strain infections of unknown etiology an attempt was made to assay the serological response in monkeys after subcutaneous inoculation with various strains of C. immitis. Arthrospores of Silveira, D-76, M-11, Cash, and CW1 strains of C. immitis were subcutaneously inoculated into monkeys as previously reported by Converse et al.[9] Serum samples were obtained from each inoculated monkey after 10 months and their antibody content was determined by the AGPI technique. These results are shown in Table 5 and demonstrate that the measured antigen-antibody reaction is not limited to infections by the Cash strain of C. immitis. This table further demonstrates the relative potency of these subcutaneously injected strains of C. immitis. Although titers occurred after injection of all five strains, these titers did not remain detectable after 10 months in the monkey groups receiving the weaker antigenic strains.

TABLE 4. COMPARISON OF SEROLOGICAL RESULTS IN MONKEY'S RESPONSE TO CUTANEOUS AND PULMONARY COCCIDIOIDOMYCOSIS[a]

Immunization		Prechallenge Titer (6 months postvaccination) Skin Test	CF Titer	AGPI Titer	15 Days After Respiratory Challenge (Silveira) CF Titer	AGPI Titer
Unvaccinated (controls)	T-40	-	-	-	-	-
	T-34	-	-	-	-	-
	T-74	-	-	-	-	-
	TR-74	-	-	-	-	-
	T-66	-	-	-	-	-
Viable vaccine (Silveira spores)						
dose 10	T-78	+	32	64	64	64
	S-22	+	-	8	8	64
	T-67	+	-	8	16	32
	T-44	+	-	4	8	32
dose 100	T-38	+	16	32	32	32
	T-30	+	-	4	8	16
	T-77	+	-	2	-	8
	T-57	+	-	4	8	16
dose 1000	T-63	+	-	32	8	32
	T-21	+	16	64	16	64
	T-17	+	-	16	-	32
	T-43	+	256	512	256	512
Nonviable vaccine (formalin-killed Cash strain)	S-38	±[b]	-	-	-	8
	S-13	±	-	-	-	4
	TR-76	±	-	-	-	2
	TR-75	±	-	-	-	32

a. Reciprocal titers are presented.
b. Questionable results (erythema but no induration).

TABLE 5. SEROLOGICAL RESPONSE OF MONKEYS TO SUBCUTANEOUS INOCULATION WITH ARTHROSPORES OF VARIOUS STRAINS OF *COCCIDIOIDES IMMITIS*[a]

Immunization Dose, spores	Strain				
	Silveira	D-76	M-11	Cash	CW1
10	1:8	1:128	1:32	Negative	1:2
	1:8	Negative	Negative	Negative	Negative
	1:64	Negative	Negative	Negative	Negative
	1:64	Negative	1:32		Negative
100	1:128	1:64[b]	1:128	Negative	Negative
	1:128	1:256	Negative	Negative	Negative
	1:512[c]	1:512	Negative	Negative	1:8
	NT[d]		1:128		

a. Each entry represents an individual monkey.
b. This monkey was immunized with formalin-killed spores prior to injection with viable D-76.
c. Disseminated infection.
d. Not tested (early death; disseminated infection).

Additionally, several human sera were obtained from known cases of histoplasmosis and blastomycosis. These sera were titrated by the AGPI procedure to determine if they contained any cross-reacting antibody that would combine with the available coccidioidin antigen and prevent tne formation of the standardized precipitin line in agar-gel, as determined by the block titration. The results of this experiment are presented in Table 6, which indicates a lack of cross-reacting antibodies in these sera to the AGPI coccidioidin. These same sera showed a cross-reacting antibody to be present when the CF test was used in their assay for coccidioidomycotic antibodies. However, all of the positive reactions were obtained with sera that showed anticomplement activity, as did some of the negative serum specimens.

E. SEROLOGICAL DIAGNOSIS OF A HUMAN LABORATORY-ACQUIRED CASE OF COCCIDIOIDOMYCOSIS USING THE AGPI TECHNIQUE

Recently a human case of laboratory-acquired coccidioidomycosis was diagnosed by isolation of *C. immitis* from the sputum and confirmed by the serological titer rise of specific antibodies using the AGPI technique. CF antibodies and C-reactive protein (CRP) were also determined on each serial

serum sample and the results are shown in Table 7. This table presents some interesting information in regard to a mild nondisseminated case of laboratory-acquired coccidioidomycosis. The C-reactive protein results indicate an inflammatory condition and parallel the clinical course of the illness. As the C-reactive protein content of the serum declined there was a corresponding increase in specific antibody titer, as first determined by the AGPI technique and later by the CF technique. The coccidioidin skin test did not convert from negative to positive until there was a definite serum antibody titer to Coccidioides.

TABLE 6. CROSS-REACTION STUDIES WITH HISTOPLASMOSIS AND BLASTOMYCOSIS HUMAN SERA

Serum	Number	Coccidioidomycosis Reciprocal Titer AGPI	CF
Histoplasmosis			
	B-13749	-	-[a]
	B-14472	-	5[a]
	B-12299	-	-
	B-13775	-	10[a]
	B-14911	-	-[a]
	B-14717	-	-
Blastomycosis			
	B-14781	-	-
	B-14414	-	5[a]
	B-14740	-	-[a]
	B-14762	-	-[a]
	B-14977	-	-
Coccidioidomycosis			
	B-10989	32	20
	B-13041	32	20[a]
	B-10468	32	40
	B-12838	32	10
Positive Human Serum Control Lot 5 (7-17-61)		64	80
Negative Human Serum Control		-	-

a. Serum still retained anticomplement activity.

TABLE 7. AGPI TITERS ON SERIAL SERUM SAMPLES FROM A LABORATORY-ACQUIRED CASE OF COCCIDIOIDOMYCOSIS

Serum Specimen Date	Hospitalization	CRP	Skin Test	Reciprocal Titer AGPI	CF
Mar 26	-75	-	-	-	-
June 9[a]	hospitalized	3+	-	-	-
11	3	9+		-	-
13	5	6+		-	-
15	7	4+		-	-
16	8	4+		-	-
18	10	4+	±	2	-
22	14	±	±	64	-
July 1	23	-		128	16
8	30	-		64	16
29[b]	51	-	+	32	4
Aug 5	58	-	+	16	4
12	65	-		32	-
19	72	-		32	4
Sept 27	111	-		8	-
Oct 18	132	-		16	4
Jan 17	223	-		16	-
Apr 17	313	-		-	-

a. Spherules with endospores in sputum; vegetative forms isolated from sputum June 21. Left apical infiltrate noted on admission.

b. Discharged from hospital July 12; diagnosis: Pulmonary coccidioidomycosis.

IV. DISCUSSION

Although the ID agar-gel test described by Huppert and Bailey[1] correlated with the CF test in 98% of the assayed sera, the latter serological procedure had to be employed to determine the specific antibody titer to coccidioidomycosis. The results of this experiment indicate that the AGPI procedure can determine titers in positive Coccidioides sera that are similar to those determined by the CF method. In certain cases of coccidioidomycosis, however, the AGPI procedure detects these titers earlier and more consistently than does the CF test. Additionally, serum specimens, often routinely shown as anticomplementary by the CF procedure, can be utilized in the AGPI test because this test does not depend on complement for its activity. Thus, the AGPI test eliminates the necessity for standardization of complement and the use of a sheep cell indicator system.

When the AGPI procedure was compared with the modified ID test for detection of Coccidioides antibodies, the results indicated a greater sensitivity by the AGPI technique because low-titered sera were not detected by the modified ID method. This was probably because there was insufficient antibody in the serum specimen to precipitate enough antigen to form a visible precipitin line. When the modified ID test did detect antibody titers, they were never of the magnitude attained by the AGPI technique. These results also indicate that if the diffusing antigen in the modified ID test is diluted, a higher titer is attained than that reached by diffusion of the concentrated antigen.

The advocated AGPI procedure derives its sensitivity in determining coccidioidomycotic antibodies by inhibition of the minimum reacting dilutions of a soluble, diffusible antigen-antibody system that is predetermined by a block titration of the reacting reagents in agar-gel plates. Variations in batch lots of the C. immitis antigen or in the antibody content of any known positive serum are minimized by this block titration.

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